

**NORMAL AND
DICOUMAROL-INDUCED PROTHROMBIN
STRUCTURE AND PROPERTIES**

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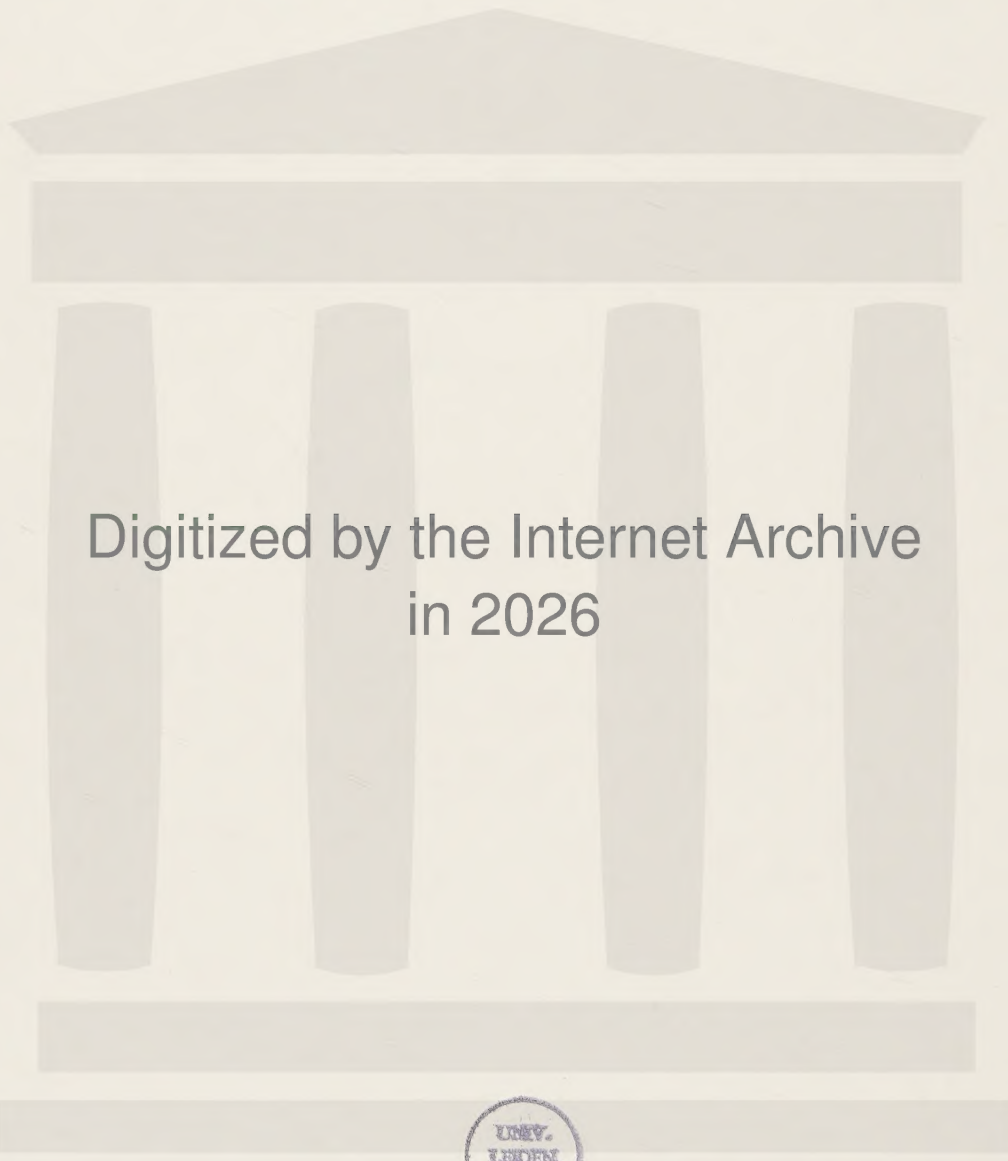
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JOHAN STENFLO
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**NORMAL AND
DICOUMAROL-INDUCED PROTHROMBIN
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MALMÖ 1973

This thesis is a summary of the following communications referred to in the text by their Roman numerals:

- I. Vitamin K and the biosynthesis of prothrombin. I. Identification and purification of a dicoumarol-induced abnormal prothrombin from bovine plasma, (1972). J. Biol. Chem., 247, 8160. In collaboration with P.O. Ganrot.
- II. Vitamin K and the biosynthesis of prothrombin. II. Structural comparison of normal and dicoumarol-induced bovine prothrombin, (1972). J. Biol. Chem., 247, 8167.
- III. Binding of Ca^{2+} to normal and dicoumarol-induced prothrombin, (1973). Biochem. Biophys. Res. Commun., 50, 98. In collaboration with P.O. Ganrot.
- IV. A conformational study of normal and dicoumarol-induced prothrombin, (1973). FEBS Letters, accepted for publication. In collaboration with I. Björk.
- V. Vitamin K and the biosynthesis of prothrombin. III. Structural comparison of an amino-terminal fragment from normal and from dicoumarol-induced bovine prothrombin.



English was revised by L. James Brown

INTRODUCTION

Prothrombin is a plasma glycoprotein, which occupies a central position in blood coagulation. It is the proenzyme of the serine protease thrombin which catalyzes the conversion of fibrinogen to fibrin by splitting four peptide bonds in fibrinogen. Thrombin has a much higher specificity than the other serine proteases and, besides its natural substrate, fibrinogen, there are only very few proteins that are digested by thrombin (Magnusson, 1971).

The enzymatic properties of thrombin, i.e. its interaction with fibrinogen and with high and low molecular weight non physiological substrates, are known in detail (Magnusson, 1971). In contrast, the events leading to the activation of prothrombin are less well understood. As early as 1890 it was recognized that calcium ions were necessary for the coagulation of blood (Arthus and Pagès, 1890). However, as shown by Hammarsten (1896) the conversion of fibrinogen to fibrin by thrombin does not require calcium ions. He showed instead that the step requiring calcium ions is the conversion of prothrombin to thrombin. In 1905 Morawitz formulated the so-called classical coagulation theory (Morawitz, 1905). According to that theory the activation of prothrombin to thrombin requires not only calcium ions, but also thromboplastin, which is a phospholipid rich fraction obtained from homogenates of brain or lung, and which is insoluble under physiological conditions. Valuable contributions have since been made to our knowledge

of the steps preceding the activation of prothrombin in blood coagulation. Thus, several coagulation factors have been identified i.e. factors V, VII, VIII, IX, X, XI and XII, all of which take part in these early steps leading to prothrombin activation (Esnouf and Macfarlane, 1968). Some of these steps take place on the surface of lipid structures. In these interactions Ca^{2+} is essential for the binding of prothrombin to the phospholipids (Barton and Hanahan, 1969, Bull et al., 1972). The mechanisms of this interaction and of prothrombin activation are, however, still obscure. This gap in our knowledge is due mainly to the fact that with the exception of factor X (Jackson, 1972, Fujikawa et al., 1972a, Fujikawa et al., 1972b, Titani et al., 1972), the coagulation factors taking part in the early steps of blood coagulation have not been characterized chemically.

In contrast with many of the other proteins involved in the blood coagulation, prothrombin and thrombin are rather well characterized, especially bovine prothrombin and thrombin, whose amino acid sequences are partly known (Magnusson, 1971). Bovine prothrombin has a molecular weight of 70,000-75,000 (Tischhoff et al., 1968, Ingwall and Scheraga, 1969) and contains 12-13 % carbohydrate (Magnusson, 1965a, Nelsestuen and Suttie, 1972a). The molecular weight of bovine thrombin is 30,000-35,000 and it contains approximately 5 % carbohydrate (Magnusson, 1971). Whereas prothrombin is a single chain structure, thrombin consists of two polypeptide chains linked together by disulfide bridges (Magnusson, 1971). The activation of prothrombin to thrombin involves cleavage of several peptide bonds. The B chain of thrombin is clearly homologous to trypsin, chymotrypsin and elastase, which suggests that prothrombin and the pancreatic serine proteases have evolved from a common ancestor (Magnusson 1971).

In 1928-1930 Dam showed that chicken fed sterol-free diets to which vitamin A and D had been added developed

haemorrhages. This was because of decreased coagulability of the blood (Dam 1929, 1930). Dam proposed the term vitamin K for the fat-soluble protective factor occurring in vegetables (Dam 1935a,b). It was soon realized that there were at least two naturally occurring forms of the vitamin, namely phylloquinone or vitamin K₁, which was isolated from alfalfa and menaquinone, or vitamin K₂ isolated from purified fish meal. The early work on vitamin K and related subjects has been reviewed by Dam (1966). The chemical structure of these two compounds was established in 1939. In 1936 it was shown by Schönheyder and Dam that the coagulation defect in the chicks was due to decreased prothrombin activity. In 1936-1938 it was demonstrated that vitamin K could correct the coagulation defect occurring in obstructive jaundice.

Not only the activity of prothrombin, but also that of other coagulation factors (VII, IX and X) is diminished in vitamin K deficiency. A property which they share with prothrombin is that they are adsorbed to barium sulfate and similar salts of divalent cations. The activated zymogen, thrombin, is not adsorbed to barium sulfate, nor are the homologous pancreatic proteases. The structural basis of this property which is shared by the vitamin K dependent coagulation factors is still unknown. Adsorption to barium citrate, for example, is a valuable step in the purification procedures of the coagulation factors belonging to this group.

Bleedings, subcutaneous and intramuscular, caused by a low prothrombin activity in the blood plasma in vitamin K deficiency can also be induced by the anticoagulant dicoumarol, 3,3-methylene-bis (4-hydroxycoumarin). This substance was isolated from spoiled clover hay by Campbell and Link (1941). Several anticoagulants with the 4-hydroxycoumarin structure have since been isolated e.g. Warfarin and Tromexan. Dicoumarol and the related anticoagulants have a potent antivitamin K activity, which can, however,

be reversed by administration of vitamin K₁. Dicoumarol has been widely used in experimental investigations of the mode of action of vitamin K. Furthermore, it has been of great value as an anticoagulant in clinical medicine. Phylloquinone oxide has approximately the same biological activity as phylloquinone in vitamin K deficient rats (Fieser et al., 1941). However, in Warfarin treated rats phylloquinone is an effective antagonist of the anticoagulant, whereas the oxide is inactive (Bell and Matschiner, 1970). It has been suggested that the anticoagulant inhibits prothrombin synthesis by inhibiting the conversion of phylloquinone oxide, known to occur in small amounts in the liver, to phylloquinone.

During the last decade serious efforts have been made by several groups to unveil the mode of action of vitamin K and of dicoumarol. As pointed out by Martius, there are essentially three different ways in which the vitamin might function. It might be a prosthetic group in the coagulation factors with a hitherto unknown mode of action. The vitamin might participate in the formation of the coagulation factors and this might be its only function. According to the third hypothesis, the vitamin takes part in the general metabolism but for some reason its deficiency first becomes manifest in the synthesis of the coagulation factors (Martius, 1966).

At present none of the above hypotheses can be ruled out completely. However, the hypothesis put forward by Martius and coworkers that vitamin K is in some way necessary in oxidative phosphorylation and that the low prothrombin activity in vitamin K deficiency is an early sign of a decreased energy supply in the cell has not been accepted by other workers in the field (Ernster et al., 1962). There does not seem to be any general involvement of vitamin K in protein synthesis (Hill et al., 1968, Berry et al., 1971). Instead, the effects of vitamin K deficiency or administration of dicoumarol so far observed have been confined to the

synthesis of clotting factors. Using the immunofluorescence technique Barnhart and Anderson (1962) have shown that the vitamin does not control the release of biologically active preformed prothrombin from the liver cells. As demonstrated by Bell and Matschiner (1969) the vitamin does not control the rate of degradation of prothrombin either.

Puromycine and cycloheximide are inhibitors of proteins synthesis acting at the ribosomal level. Recent papers describe the use of these inhibitors in experiments designed to establish whether the regulatory mechanism of vitamin K is exerted at the ribosomal level, regulating the intrinsic rate of the ribosomal synthesis of these proteins, or at some hitherto undefined post-ribosomal level. In most of these studies prothrombin has been assayed by its biological activity and not with immunochemical methods. Studies of this kind are known to be difficult to compare and to interpret and have also yielded conflicting results. Thus, some workers hold the opinion that vitamin K regulates the rate of de novo synthesis of prothrombin and more specifically controls some event in the initiation of ribosomal prothrombin synthesis (Li et al., 1970, Johnston and Olsson, 1972). However, recent evidence produced by several groups and obtained with different methods favours the opinion that vitamin K is necessary for the modification of the prothrombin polypeptide chain in some postribosomal event (Hill et al., 1968, Bell and Matschiner, 1969, Suttie, 1970, Pereira and Couri, 1971). Thus Shah and Suttie (1971) have shown that administration of vitamin K to vitamin K deficient rats treated with cycloheximide raised the prothrombin activity, in one hour to about 70 % of the activity in rats not treated with cycloheximide. When radioactive amino acids were given to the rats after treatment with the cycloheximide the newly formed prothrombin was found to contain no radioactivity. This strongly suggests involvement of vitamin K in the conversion of a prothrombin

precursor to biologically active prothrombin.

Hemker and coworkers (1963) have found an inhibitor of prothrombin activation in blood plasma from persons with vitamin K deficiency. They postulated that this inhibitor was a precursor of prothrombin and converted to biologically active prothrombin in a vitamin K dependent step. In 1968 it was demonstrated (Niléhn and Ganrot, 1968, Ganrot and Niléhn, 1968, Josso et al., 1968) that blood plasma from persons treated with dicoumarol contained a protein which has the same main antigenic determinants as normal prothrombin, but which lacks prothrombin activity. Later this protein was also found in blood plasma from persons with vitamin K deficiency (Ganrot and Niléhn, 1971). It was also shown by Ganrot and Niléhn that normal prothrombin binds Ca^{2+} , whereas abnormal prothrombin does not. Furthermore, this abnormal prothrombin, in contrast with normal prothrombin, was not adsorbed to barium citrate and was not converted to thrombin on coagulation of the plasma, but appeared unchanged in the serum. This abnormal prothrombin is probably a precursor of normal prothrombin, but unequivocal proof is still lacking. The above investigations lent very strong support to the opinion that vitamin K exerts its effect in a postribosomal step and does not regulate the rate of de novo synthesis of prothrombin. Recently the BaSO_4 adsorbable proteins in rat plasma from normal and from vitamin K deficient animals were investigated by Johnson et al. (1972). When the BaSO_4 eluate was analyzed by polyacrylamide gel electrophoresis it was shown that the prothrombin band decreased in intensity in vitamin K deficiency, but in plasma from the deficient animals there appeared a new protein band whose intensity varied inversely with that of prothrombin and which had no prothrombin activity. The relationship, if any, between this protein and a prothrombin was not established.

According to a recent hypothesis, vitamin K is in-

volved in the biosynthesis of the carbohydrate portion of prothrombin (Johnson et al., 1971, Pereira and Couri, 1971). However experiments which have shown that prothrombin devoid of a substantial amount of its carbohydrate has normal prothrombin activity (Nelsestuen and Suttie, 1971) and binds calcium ions (Stenflo, 1970) argues against such an assumption (see discussion of papers II and V).

PRESENT INVESTIGATION

The presence of an abnormal prothrombin in the plasma from dicoumarol treated oxen was established (I). Normal prothrombin was purified from bovine plasma, and a method was devised for the purification of the abnormal prothrombin (I). The two prothrombins were characterized and their primary structures were compared with conventional methods (II). The calcium binding characteristics of the two prothrombins were investigated with equilibrium dialysis (III). Their conformation, both with and without bound Ca^{2+} , was compared with immunochemical and spectroscopic methods as conformational probes (II, III and IV). A fragment with different Ca^{2+} binding properties was isolated from thrombin digests of the two prothrombins. The antigenic properties and the primary structure of these two fragments were compared (V). The following aspects of the work are discussed in this thesis:

The identification and purification of dicoumarol-induced prothrombin.

The structural comparison of normal and dicoumarol-induced prothrombin.

Studies on the Ca^{2+} binding of the two prothrombins and a comparison of their conformation with and without bound Ca^{2+} .



Fig. 1. Crossed immunoelectrophoresis pattern of bovine plasma in agarose gel. Barbitol buffer pH 8.6, 2 mM in calcium lactate. Above, sample obtained before administration of dicoumarol. The three samples below were obtained 2; 4 and 7 days after the beginning of dicoumarol administration. The prothrombin activity of the samples relative to a standard is from above 140 %; 94 %; 49 %; and 22 %.

THE IDENTIFICATION AND PURIFICATION OF DICOUMAROL-
INDUCED PROTHROMBIN FROM BOVINE PLASMA (I)

A structural comparison of normal and dicoumarol-induced prothrombin required large amounts of the purified proteins. Since the prothrombin concentration in blood plasma is 50-100 mg per liter, large volumes of plasma were required as starting material for the purification. Human plasma could be obtained only in limited quantities and therefore bovine plasma was used. An advantage of bovine material was that well established methods were available for the purification of normal bovine prothrombin and that the structure of bovine prothrombin is known much better than the structure of prothrombin from other species (Magnusson, 1971).

Two oxen, periodically treated with dicoumarol supplied the plasma used for the purification of the abnormal prothrombin, whereas the normal prothrombin was purified from plasma obtained from the slaughter-house. To ascertain that there were no species differences between human and bovine plasma with regard to the appearance and properties of an abnormal dicoumarol-induced prothrombin the electrophoretic and immunochemical properties of prothrombin in samples obtained before and during dicoumarol administration to the oxen were investigated with crossed immunoelectrophoresis using rabbit antibovine prothrombin. These studies established that

during dicoumarol treatment there appears in the plasma an abnormal prothrombin, whose concentration varies inversely with that of normal prothrombin (Fig. 1). From the electrophoretic mobility of the two prothrombins in buffers containing either Ca^{2+} or EDTA it was inferred that normal prothrombin binds Ca^{2+} , whereas dicoumarol-induced prothrombin does not. It was also shown that barium citrate adsorption of plasma removed normal prothrombin, whereas the abnormal prothrombin appeared to remain quantitatively in the supernatant. Moreover, in contrast with normal prothrombin the abnormal prothrombin remained apparently unchanged in plasma samples that had been coagulated. These results are identical with those obtained in human material by Ganrot and Niléhn (1968). It has recently been claimed that bovine plasma contains three abnormal dicoumarol-induced prothrombins (Malhotra, 1972a). With our immunochemical techniques it was never possible to observe more than one.

Normal prothrombin was purified with the method of Moore et al. (1965) as modified by Ingwall and Scheraga (1969). This method utilises adsorption of the prothrombin to barium citrate and subsequent elution with citrate, which gives an excellent initial purification. Since the bulk of the abnormal prothrombin was not adsorbed to barium citrate, column chromatography had to be resorted to for the purification of this protein. A good supply of the pure proteins is a prerequisite for a structural comparison of them. It was therefore necessary to design the initial purification steps so that the entire procedure got a high capacity. A useful property of the abnormal prothrombin was that the ionic strength required for elution of it from DEAE cellulose and DEAE Sephadex was very high.

The purification procedure for the dicoumarol-induced prothrombin is summarized in Table I. The first step involved removal of normal prothrombin by adsorption to

Table I

Purification of dicoumarol-induced prothrombin

Fraction		Protein	Prothrombin ^{a)}	Recovery	Purification
		mg		%	-fold
I	Bovine plasma ^{b)}	200 000	140	100	1
II	Ammonium sulfate	51 000	100	72	2.8
III	DEAE-cellulose	2 600	67	46	37
IV	DEAE-Sephadex	620	55	38	130
V	Hydroxylapatite	170	33	23	280
VI	Sephadex G-100	36	26	19	1000

a) Arbitrary units

b) After removal of normal prothrombin by adsorption to BaSO₄

barium citrate and was followed by ammonium sulfate fractionation. The following step was a displacement chromatography on DEAE cellulose. This step was easily monitored since no prothrombin appeared in the effluent until most of the column had been coloured blue by adsorbed ceruloplasmin. The proteins were eluted from the column with 0.3 M KH₂PO₄. In a later modification of the purification procedure (V) elution was accomplished with a combined pH and ionic strength gradient. This made it possible to process even larger amounts of material without increasing the size of the gel beds in the following chromatographies. After this modification omission of the hydroxylapatite chromatography resulted in preparations with only small amounts of contaminating proteins.

The homogeneity of the normal and the dicoumarol-induced prothrombin was tested with several methods. Thus both prothrombins showed symmetrical boundaries in sedimentation velocity experiments (II). Polyacrylamide disc gel electrophoresis in buffer without dissociating agents did not reveal any inhomogeneity. Neither did electro-

phoresis in polyacrylamide gel containing 8 M urea at two pH values nor polyacrylamide gel electrophoresis in sodium dodecylsulfate (II). Analysis of the NH_2 -terminal amino acid gave only one residue (alanine) whether the phenylisothiocyanate method or the Dansyl method was used (II and V). The properties of the purified normal and dicoumarol-induced prothrombin with respect to Ca^{2+} binding and immunochemical properties were identical with those observed in unfractionated plasma. The purified normal prothrombin, as in unfractionated plasma, had a high one stage prothrombin activity, whereas the dicoumarol-induced prothrombin had no discernible activity. No absolute criteria exist which can ascertain that purified proteins are native. However the results of the comparison of the properties of the two prothrombins in unfractionated plasma and in the purified state shows that both proteins when purified retain their characteristic properties, which indicates that both are native.

The purified dicoumarol-induced prothrombin did not interfere with the activation of normal prothrombin. This is noteworthy since Hemker et al. (1963) proposed that during dicoumarol administration a precursor of prothrombin is synthesized which acts as a competitive inhibitor of the prothrombin activation.

Dicoumarol-induced bovine prothrombin has recently been purified by Nelsestuen and Suttie (1972b) with a method similar to the one described above. However, their method did not involve ammonium sulfate fractionation and hydroxylapatite chromatography, but acid precipitation. The overall yield appears to be similar to that obtained with our method. Their data indicate that some of the abnormal prothrombin is lost in the barium citrate adsorption step. Since crossed immunoelectrophoresis, which was used by us to monitor the removal of normal prothrombin in the barium citrate adsorption, is only semiquanti-

tative, small losses would not have been detected. However, it should be noted that quantitative determinations of the total amount of prothrombin with immunochemical methods in mixtures of the two are unreliable because of the difference in their antigenic properties.

Malhotra and Carter (1971) have purified prothrombin from dicoumarol treated steers and shown that it has a specific activity that is lower than that of normal prothrombin. Later this material was separated into two components, one of which probably consisted of normal prothrombin while the other might be identical with the abnormal prothrombin studied by us (Malhotra, 1972b).

STRUCTURAL COMPARISON OF NORMAL AND DICOUMAROL-INDUCED PROTHROMBIN (II and V)

As a first step in a comparison of the chemical and physical properties of normal and dicoumarol-induced prothrombin a characterization of the latter was desirable. Normal prothrombin is well characterized and parts of its amino acid sequence is known (Magnusson, 1971). In the present investigation normal prothrombin was therefore included as a reference.

The molecular weight of prothrombin has been reported to be between 70,000 and 75,000 (Tishkoff et al., 1968, Ingwall and Scheraga, 1969). With gel electrophoresis in sodium dodecyl sulfate a value of 72,000 was obtained. Mixtures of normal and dicoumarol-induced prothrombin migrated as a single band indicating that both consist of one polypeptide chain and that they have identical molecular weights. This result was confirmed by Nelsestuen and Suttie (1972b) with the same technique. Sedimentation velocity analysis gave a S_{20}^0 of 4.9 for normal prothrombin, which is in agreement with values reported earlier (Magnusson, 1971). The sedimentation velocities of the two prothrombins were identical. Determination of Stoke's molecular radius gave a value of 40-41 Å for both indicating that they have identical diffusion coefficients. Furthermore, the two prothrombins had identical amino acid and carbohydrate compositions (see below)

and therefore identical partial specific volumes. Since their molecular weights can be calculated from these data the results confirm that the two prothrombins have identical molecular weights.

Quantitative amino acid analysis of the two prothrombins gave no evidence of differences in amino acid composition. The results were in fair agreement with earlier published values (Tishkoff et al., 1968, Ingwall and Scheraga, 1969, Cox and Hanaham, 1970) except for the half-cystine and methionine values which were somewhat higher. There was, however, fair agreement between the results of a separate sulfur determination and the sulfur content calculated from the amino acid analysis. The differences in Ca^{2+} binding between the two prothrombins warranted a quantitative determination of the number of free carboxyl groups, since they are known to be essential for the binding of this ion. This was performed with the method of Hoare and Koshland (1972), which involves activation of free carboxyl groups with a water soluble carbodiimide and subsequent reaction with glycine methylester resulting in incorporation of glycine. The number of carboxyl groups, as determined with this method, was approximately 75 in both proteins. The Ca^{2+} binding might also be accounted for by ester bound phosphate which however was ruled out by separate phosphorous determinations.

Quantitative carbohydrate analysis gave a total carbohydrate content of 13.6 % for normal prothrombin and 14.5 % for dicoumarol-induced prothrombin. In both prothrombins the carbohydrate portion consisted of mannose, galactose, N-acetylglucosamine and sialic acid. There were no quantitative differences between the two prothrombins that could not be accounted for by experimental error. Nelsestuen and Suttie (1972b) have arrived at the same conclusion. However, these results do not preclude small structural differences in the carbohydrate part of the molecules. It has been de-

monstrated by Nelsestuen and Suttie (1971) that desialylated normal prothrombin has a normal prothrombin activity and by Stenflo (1970) that the difference in Ca^{2+} binding between normal and dicoumarol-induced prothrombin is uninfluenced by desialylation. This evidence seems to argue strongly against the differences in properties between the two prothrombins being caused by differences in the carbohydrate portion of the molecules.

One mole of alanine was obtained as the NH_2 -terminal amino acid in both prothrombins with the phenylisothiocyanate method. Dansyl-Edman degradation yielded the NH_2 -terminal sequence Ala-Asx-Lys- in both. This is in agreement with earlier results (Magnusson, 1965b). An attempt to determine the COOH-terminal amino acid with hydrazinolysis was made since, as reported by Magnusson and Steele (1965), carboxypeptidase does not liberate any amino acid. Serine was obtained repeatedly in both prothrombins. On the basis of indirect evidence Magnusson (1969) has reported that serine is the COOH-terminal amino acid in normal prothrombin. Malhotra (1972c) has recently obtained glutamic acid or glutamine as COOH-terminal amino acid in bovine prothrombin. No explanation can be offered for this discrepancy.

Peptide maps were prepared from the completely reduced and aminoethylated normal and dicoumarol-induced prothrombin. There were no reproducible differences between the peptide maps in several experiments. The two prothrombins behaved identically in isoelectric focusing in 8 M urea. Furthermore, no separation was obtained between the reduced and alkylated prothrombins on electrophoresis in polyacrylamide gel containing 8 or 9M urea at pH 8.9 or at pH 2.7. However, with intact disulfide bonds the two prothrombins differed in electrophoretic mobility in polyacrylamide gel containing 8 M urea at pH 8.9. Under these conditions separation was also obtained between their cyanogen bromide fragments. These results

were compatible with a minor structural difference between the two prothrombins. To account for the difference in electrophoretic mobility between them with intact disulfide bonds and for the unsuccessful attempts to distinguish between them when reduced and alkylated both by peptide mapping and by polyacrylamide gel electrophoresis in 8 M urea, the hypothesis was advanced that there is a difference in the pairing of the half-cystine residues. The fact that vitamin K can function as an oxidation - reduction mediator in the cell made this hypothesis very attractive.

Josso (1968) and coworkers have shown that the dicoumarol-induced prothrombin, like normal prothrombin is activated by staphylocoagulase and Nelsestuen and Suttie (1972b) have reported that the abnormal prothrombin gives rise to a normal amount of thrombin when activated by nonphysiological activators such as trypsin. Since normal Ca^{2+} binding seems to be essential for the physiological activation of prothrombin, it was reasonable to infer that the Ca^{2+} binding groups are located in that part of the molecule which is split off during activation to thrombin. Furthermore, the structural difference between the two prothrombins is probably to be found in this part of the molecule. Thus it was desirable to find a method to degrade the two prothrombins into smaller fragments, if possible with the Ca^{2+} binding groups intact and on one fragment only. Digestion with thrombin proved to be suitable in that it degraded the two prothrombins into two fragments, the smaller of which had a molecular weight of approximately 27,000. The fragments obtained from the normal and the dicoumarol-induced prothrombin had identical molecular weights. The larger fragment, which upon further cleavage, gives rise to thrombin (Stenn and Blout, 1972) had identical electrophoretic and immunochemical properties in both prothrombins. The small fragment from normal prothrombin had a Ca^{2+} dependent electrophoretic

mobility resembling that of intact normal prothrombin, whereas the electrophoretic mobility of the corresponding fragment from the dicoumarol-induced prothrombin was independent of the Ca^{2+} concentration.

The prothrombin fragments were separated on a preparative scale by gel filtration on Sephadex G 100. Since the small fragments produced by thrombin digestion differed with respect to Ca^{2+} binding, the subsequent structural comparison was restricted to them. The NH_2 -terminal amino acid sequence of the small fragment from both prothrombins was Ala-Asx-Lys-Gly-Phe-Leu-, which indicates that they occupy the NH_2 -terminal part of the intact proteins which were found to have the sequence Ala-Asx-Lys-. As with the intact normal and the dicoumarol-induced prothrombin, no difference in amino acid composition was found. The total carbohydrate content was approximately 21 % for both. The carbohydrate consisted of mannose, galactose, N-acetyl-glucosamine and sialic acid. There was no quantitative difference in composition between the fragment from the normal and the dicoumarol-induced prothrombin. This argues further against the assumption that vitamin K is necessary for the glycosylation of the prothrombin polypeptide chain and that this process is inhibited by dicoumarol.

Peptide mapping experiments were performed on the small fragment from the normal and the dicoumarol-induced prothrombin. There was no obvious difference between peptide maps prepared from tryptic digests of the completely reduced and aminoethylated proteins. On the other hand, thermolysin digests of the fragments with intact disulfide bridges gave peptide maps with clearcut differences. These differences were also evident in peptide maps of thermolysin digests of the completely reduced and aminoethylated proteins (Fig. 2). These results indicate that, in contrast with the hypothesis advanced above, different pairing of half-cystine residues, if any,

cannot be the only covalent difference between the two prothrombins. Instead, the results suggest a modified amino acid residue or the presence of a prosthetic group linked with an acid labile bond as also suggested by Nelsestuen and Suttie (1972b,c).

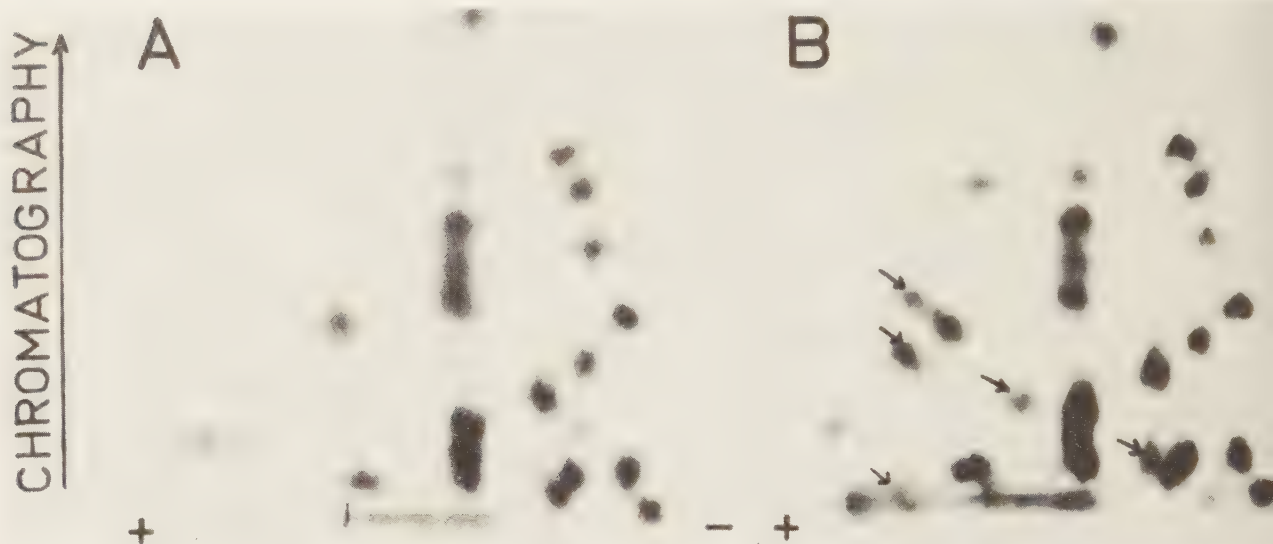


Fig. 2. Peptide map of thermolysin digest of completely reduced and aminoethylated small fragments from thrombin digests of normal (A) and dicoumarol-induced prothrombin (B). The arrows denote the peptides present in one of the peptide maps but not in the other.

THE CONFORMATION AND Ca^{2+} BINDING PROPERTIES OF NORMAL
AND DICOUMAROL-INDUCED PROTHROMBIN (II, III, IV and V)

Except for their biological activity and Ca^{2+} binding characteristics the properties of normal and dicoumarol-induced prothrombin are very similar. Thus no difference was found between them on titration of phenolic groups or determination of fluorescence emission spectra. However normal prothrombin has a somewhat higher electrophoretic mobility than abnormal prothrombin. They can also be distinguished by quantitative immunoprecipitation with rabbit antiovine prothrombin raised against normal prothrombin (Fig. 3). Surprisingly enough, the dicoumarol-induced prothrombin precipitated more antibody in the equivalence zone than did normal prothrombin when the buffer contained EDTA despite the fact that the antiserum had been raised against normal prothrombin. A plausible explanation for this result was that normal prothrombin lost antigenic determinants when Ca^{2+} was removed. This was also shown to be the case, since when Ca^{2+} was included in the buffer instead of EDTA, normal prothrombin precipitated more antibody in the equivalence zone than did dicoumarol-induced prothrombin. The precipitation curves obtained with the latter protein were not influenced by the presence or absence of Ca^{2+} .

With optical rotatory dispersion (ORD) and circular

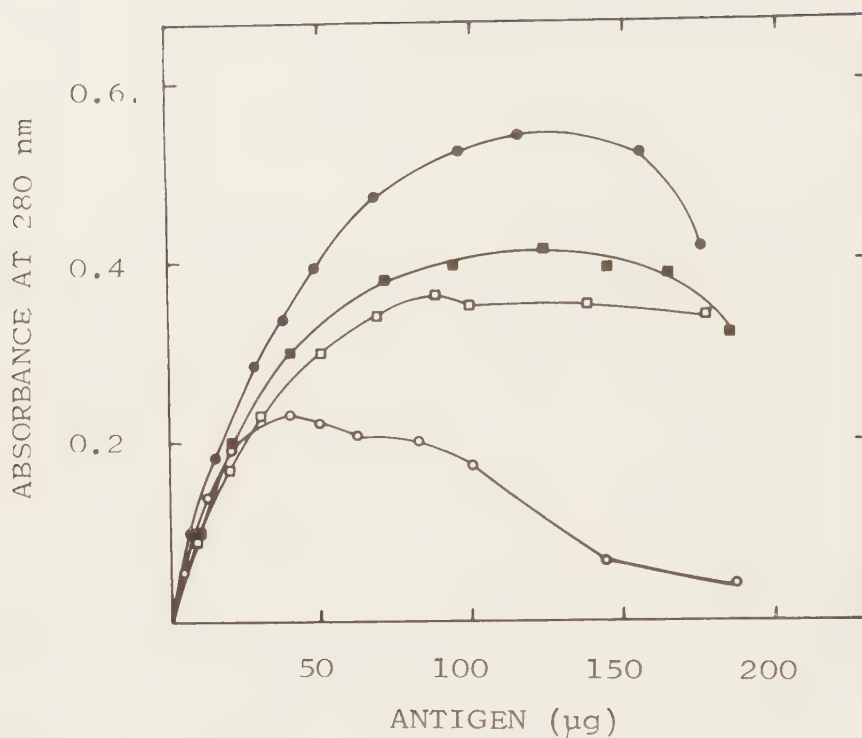


Fig. 3. Comparison of the quantitative precipitin curves of normal and dicoumarol-induced prothrombin in the presence of EDTA or Ca^{2+} . (○—○), normal prothrombin with EDTA; (□—□), dicoumarol-induced prothrombin with EDTA; (○—○), normal prothrombin with Ca^{2+} ; (□—□), dicoumarol-induced prothrombin with Ca^{2+} .

dichroism (CD) as conformational probes no evidence of any difference was found between Ca^{2+} free normal and dicoumarol-induced prothrombin. However, with Ca^{2+} in the buffer the two prothrombins behaved differently from one another. Thus, while the CD spectrum of the dicoumarol-induced prothrombin was uninfluenced by Ca^{2+} , the CD spectrum of the normal prothrombin with Ca^{2+} in the buffer indicated that this ion induced a conformational change in normal prothrombin. Since the change was manifest only in the aromatic region of the spectrum, it is probably only a local change in the neighbourhood of some aromatic amino acid. This ligand induced conformational change was not observed on titration of phenolic groups. In this context the versatility of the immuno-

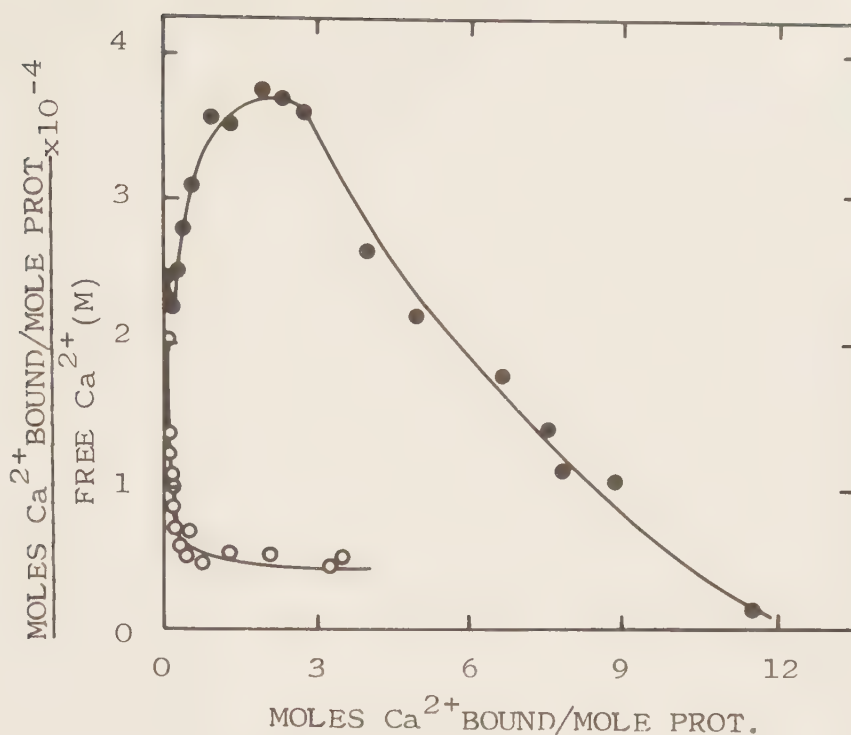


Fig. 4. Scatchard plot of Ca^{2+} binding by normal and dicoumarol-induced prothrombin. ●, Normal prothrombin; ○, dicoumarol-induced prothrombin.

chemical method as a conformational probe should be noted. Of the above methods, quantitative immunoprecipitation was the only one that could distinguish between dicoumarol-induced prothrombin and normal prothrombin both with and without bound Ca^{2+} . The usefulness of immunochemical methods can be increased still more by fractionation of the antiserum into populations of antibodies directed at specific antigenic determinants in the protein to be studied (Sachs et al., 1972a,b).

The observation that Ca^{2+} induced a conformational change in normal prothrombin suggested that its Ca^{2+} binding characteristics might be complex. To study the Ca^{2+} binding of normal and dicoumarol-induced prothrombin, in greater detail a modification of conventional equilibrium dialysis, the flow - dialysis method of Colowick and Womack (1969) was used. With this method an experiment which gave all the data necessary for a Scatchard plot could be ob-

tained in approximately half an hour, which considerably reduced the risk of autoactivation of the normal prothrombin compared with conventional equilibrium dialysis, which usually requires several hours to reach equilibrium.

The Scatchard plot of experiments on Ca^{2+} binding to normal prothrombin deviated from linearity indicating that the binding sites were not identical and independent (Fig. 4). Deviation from linearity in which the curve is concave upwards is often observed and usually attributed to heterogeneity of binding sites, but may also be caused by a negative cooperativity in the binding of the ligand. However, in the experiments with prothrombin the curve was concave downwards, which indicates a positive cooperativity (Koshland, 1970, Cook and Koshland, 1970). From a Hill plot of the same data it was inferred that the positive cooperativity was limited to the first three binding sites. The total number of binding sites was approximately ten. In contrast herewith the dicoumarol-induced prothrombin showed at most one high affinity binding site and several very low affinity binding sites, probably nonspecific. Nelstuen and Suttie (1972c) in a recent study on the Ca^{2+} binding properties of normal and dicoumarol-induced prothrombin got the same results except that they only found four binding sites in normal prothrombin. They also found a strong dependence of Ca^{2+} binding on pH with maximum binding between pH 7.5 and 9.5. A reasonable explanation for the ligand induced conformational change and the positive cooperativity in normal prothrombin would be that the first Ca^{2+} is bound to carboxyl groups in different parts in the primary structure which, by the folding of the peptide chain, can be brought in juxtaposition to each other to form a Ca^{2+} binding site. If this process brings another pair of carboxyl groups in the proximity of each other, which may give rise to electrostatic repulsion a new binding site may be formed which has a higher association constant than the first one.

The Ca^{2+} binding groups were found in the NH_2 -terminal

fragment produced by thrombin digestion of normal prothrombin, whereas the corresponding fragment from dicoumarol-induced prothrombin did not bind Ca^{2+} as judged from the electrophoretic mobility in buffer containing Ca^{2+} or EDTA. The ligand dependence of the antigenic determinants in the fragment from normal prothrombin was striking in that in crossed immunoelectrophoresis an antiserum raised against normal bovine prothrombin did not recognize the fragment when the buffer contained EDTA. On the other hand, with Ca^{2+} bound to the fragment a strong immunoprecipitate was formed. The fragment from dicoumarol-induced prothrombin gave rise to a hazy precipitate both with and without Ca^{2+} in the medium.

In blood plasma the Ca^{2+} concentration is such that the conformation of normal prothrombin, which occurs with Ca^{2+} bound is presumably always maintained. The above described conformational transition therefore appears to have no physiological implications. However, the Ca^{2+} binding is essential for proper activation of prothrombin, since these ions seem to mediate the binding of normal prothrombin to carboxyl groups in the lipoproteins and cell membranes (Bull et al., 1972). It is not known whether the latter process leads to conformational changes in normal prothrombin. At present it is reasonable to assume that dicoumarol-induced prothrombin is not activated in physiological systems because of deficient Ca^{2+} binding and thereby inability to bind to the lipid structures.

SUMMARY

Prothrombin is the proenzyme of the serine protease thrombin. The prothrombin activity in blood and the activity of three other coagulation factors (VII, IX and X) is depressed in vitamin K deficiency, leading to a decreased coagulability of the blood. The effect of administration of the vitamin K antagonist dicoumarol is identical with that observed in vitamin K deficiency. Its involvement in the biosynthesis of these coagulation factors is the only defined function of vitamin K in higher animals. Prothrombin and the three other vitamin K dependent coagulation factors share the property of being adsorbed to barium citrate. It is not known why the biosynthesis of these coagulation factors is affected by vitamin K and not the biosynthesis of other plasma glycoproteins. From studies employing inhibitors of protein synthesis it has been inferred that vitamin K is involved in a postribosomal step of the biosynthesis of the above coagulation factors.

In this thesis the appearance in bovine plasma of an abnormal prothrombin induced by dicoumarol is reported. In comparison with normal prothrombin, the abnormal prothrombin had a reduced Ca^{2+} binding capacity, it was not adsorbed to barium citrate, it had no prothrombin activity but it had the same main antigenic determinants as normal prothrombin. The concentration of the abnormal pro-

thrombin varied inversely with the concentration of normal prothrombin. A purification procedure was devised for the abnormal prothrombin whereas normal prothrombin was purified with already existing methods.

Prothrombin, which is best characterized of the vitamin K dependent coagulation factors, has a molecular weight of approximately 72,000, consists of one polypeptide chain and has a carbohydrate content of approximately 13 %. No prosthetic group other than the carbohydrate has been identified. In the structural comparison of normal and dicoumarol-induced prothrombin no difference was found between them with respect to molecular weight, amino acid and carbohydrate composition, NH_2 -terminal and COOH -terminal amino acid. Furthermore, peptide maps prepared from tryptic digests of the completely reduced and aminoethylated proteins were identical indicating that any covalent difference between them is only very small.

With quantitative immunoprecipitation it proved possible to distinguish between the two prothrombins using an antiserum raised against normal bovine prothrombin. Furthermore in contrast with dicoumarol-induced prothrombin, normal prothrombin turned out to have Ca^{2+} dependent antigenic determinants. Without Ca^{2+} in the buffer circular dichroism could not distinguish between normal and dicoumarol-induced prothrombin. However, the spectrum of normal prothrombin with bound Ca^{2+} clearly indicated that Ca^{2+} induced a conformational change in normal prothrombin, whereas the spectrum of the dicoumarol-induced prothrombin was unaffected of Ca^{2+} . Equilibrium dialysis studies showed that normal prothrombin has approximately ten Ca^{2+} binding sites. Of these, three are high affinity sites which exhibit positive cooperativity. In contrast, dicoumarol-induced prothrombin has only one high affinity binding site.

The inability of dicoumarol-induced prothrombin to activate in physiological systems is probably due to an inadequate Ca^{2+} binding. Thrombin digestion of the two prothrombins gave two fragments from each protein. The NH_2 -terminal fragment had a molecular weight of approximately 27,000 from both. This fragment from normal prothrombin bound Ca^{2+} and had Ca^{2+} dependent antigenic determinants. The corresponding fragment from dicoumarol-induced prothrombin did not bind Ca^{2+} . The amino acid and carbohydrate composition of the two fragments was identical. Peptide maps were prepared from thermolysin digests of the fragments both with intact disulfide bonds and completely reduced and aminoethylated. Clear-cut differences were observed in the peptide maps of both types of digests. This indicates that the difference between the two prothrombins consists of a modified amino acid residue or a prosthetic group, presumably linked with an acid labile bond. This structural difference probably gives rise to the conformational difference and the different Ca^{2+} binding properties of the two prothrombins.

REFERENCES

- Arthus, M., and Pagès, C. (1890) Arch. Physiol. norm. pathol. 2, 739
- Barnhart, M.I., and Anderson, G.F. (1962) Biochem. Pharmacol. 9, 23
- Barton, P.G., and Hanahan, D.J. (1969) Biochim. Biophys. Acta. 187, 319
- Bell, R.G., and Matschiner, J.T. (1969) Arch. Biochem. Biophys. 135, 152
- Bell, R.G., and Matschiner, J.T. (1970) Arch. Biochem. Biophys. 141, 473
- Berry, D.R., Philipps, G.R., and Olson, R.E. (1971) Biochem. Pharmacol. 20, 853
- Bull, K.R., Jevons, S., and Barton, P.G. (1972) J. Biol. Chem. 247, 2747
- Campbell, H.A., and Link, K.P. (1941) J. Biol. Chem. 138, 21
- Colowick, S.P., and Womack, F.C. (1969) J. Biol. Chem. 244, 774
- Cook, R.A., and Koshland, D.E. Jr. (1970) Biochemistry 9, 3337
- Cox, C.A., and Hanahan, D.J. (1970) Biochim. Biophys. Acta 207, 49
- Dam, H. (1929) Biochem. Z. 215, 475
- Dam, H. (1930) Biochem. Z. 220, 158
- Dam, H. (1935a) Nature 135, 652
- Dam, H. (1935b) Biochem. J. 29, 1273
- Dam, H. (1966) Vitamines and Hormones 24, 295
- Ernster, L., Danielsson, L., and Ljunggren, M. (1962) Biochim. Biophys. Acta 58, 171
- Esnouf, M.P., and Macfarlane, R.G., (1968) Advances in Enzymology Vol. 30, 255

- Fieser, L.F., Tishler, M., and Sampson, W. (1941) J. Biol. Chem. 137, 659
- Fujikawa, K., Legay, M.E., and Davie, E.W. (1972a) Biochemistry 11, 4882
- Fujikawa, K., Legay, M.E., and Davie, E.W. (1972b) Biochemistry 11, 4892
- Ganrot, P.O., and Niléhn, J.E. (1968) Scand. J. Clin. Lab. Invest. 22, 23
- Hammarsten, O. (1896) Z. Physiol. Chem. 22, 333
- Hemker, H.C., Veltkamp, J.J., Hensen, A., and Loeliger, E.A. (1963) Nature 200, 589
- Hill, R.B., Gaetani, S., Paolucci, A.M., RamaRao, P.B., Alden, R., Ranhotra, G.S., Shah, D.V., Shah, V.K., and Johnson, B.C. (1968) J. Biol. Chem. 243, 3930
- Hoare, D.G., and Koshland, D.E.Jr. (1967) J. Biol. Chem. 242, 2447
- Ingwall, J.S., and Scheraga, H.A. (1969) Biochemistry 8, 1860
- Jackson, C.M. (1972) Biochemistry 11, 4873
- Johnson, H.V., Martinovic, J., and Johnson, B.C. (1971) Biochem. Biophys. Res. Commun. 43, 1040
- Johnston, M.F.M., and Olson, R.E. (1972) J. Biol. Chem. 247, 4001
- Josso, F., Lavergne, J.M., Gouault, M., Prou-Wartelle, O., and Soulier, J.P. (1968) Thromb. Diath. Haemorrh. 20, 88
- Koshland, D.E. Jr. (1970) The Enzymes, Third Edition Vol. 1, 342
- Li, L.F., Kipfer, R.K., and Olson, R.E. (1970) Arch. Biochem. Biophys. 137, 494
- Magnusson, S. (1965a) Ark. Kemi 23, 285
- Magnusson, S. (1965b) Ark. Kemi 24, 375
- Magnusson, S., and Steele, B. (1965) Ark. Kemi 24, 359

- Magnusson, S. (1969) *Biochem. J.* 115, 2P
- Magnusson, S. (1971) *The Enzymes*, Third Edition Vol. 3, 277
- Malhotra, O.P., and Carter, J.R. (1971) *J. Biol. Chem.* 246, 2665
- Malhotra, O.P. (1972a) *Nature* 239, 59
- Malhotra, O.P. (1972b) *Life Sci.* 11, 901
- Malhotra, O.P. (1972c) *Life Sci.* 11, 455
- Martius, C. (1966) *Vitamines and Hormones* 24, 441
- Moore, H.C., Lux, S.E., Malhotra, O.P., Bakerman, S., and Carter, J.R. (1965) *Biochim. Biophys. Acta* 111, 174
- Morawitz, P. (1905) *Ergebn. Physiol.* 4, 307
- Nelsestuen, G.L., and Suttie, J.W. (1971) *Biochem. Biophys. Res. Commun.* 45, 198
- Nelsestuen, G.L., and Suttie, J.W. (1972a) *J. Biol. Chem.* 247, 6096
- Nelsestuen, G.L., and Suttie, J.W. (1972b) *J. Biol. Chem.* 247, 8176
- Nelsestuen, G.L., and Suttie, J.W. (1972c) *Biochemistry* 11, 4961
- Niléhn, J.E., and Ganrot, P.O. (1968) *Scand. J. Clin. Lab. Invest.* 22, 17
- Pereira, M., and Couri, D. (1971) *Biochim. Biophys. Acta* 237, 348
- Sachs, D.H., Schechter, A.N., Eastlake, A., and Anfinsen, C.B. (1972a) *J. Immunol.* 109, 1300
- Sachs, D.H., Schechter, A.N., Eastlake, A., and Anfinsen, C.B. (1972b) *Proc. Nat. Acad. Sci. U.S.A.* 69, 3790
- Shah, D.V., and Suttie, J.W. (1971) *Proc. Nat. Acad. Sci. U.S.A.* 68, 1653
- Stenflo, J. (1970) *Acta Chem. Scand.* 24, 3762

Stenn, K.S., and Blout, E.R. (1972) *Biochemistry* 11,
4502

Suttie, J.W. (1970) *Arch. Biochem. Biophys.* 141, 571

Tishkoff, G.H., Williams, L.C., and Brown, D.M. (1968)
J. Biol. Chem. 243, 4151

Titani, K., Hermodson, M.A., Fujikawa, K., Ericsson,
L.H., Walsh, K.A., Neurath, H., and Davie, E.W.
(1972) *Biochemistry* 11, 4899

